Antiurease, Antiphosphodiesterase and Antiglycation Studies of Pd(II) Complexes with Monodentate Hydrazides

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Summary: The present study was aimed to synthesize and characterize a series of Pd(II)-benzohydrazide complexes with subsequent high throughput screening to seek their effects as enzyme inhibitors and antiglycating agents. Based on complete characterization *via* elemental (CHN, Pd) analysis, physical (conductivity, magnetic moment) measurements and spectral (FT-IR, ^{1}H-NMR, ^{13}C-NMR) techniques, all Pd(II) complexes were identified as diamagnetic, neutral and orienting in trans square planar geometry with general formula $[PdL_2Cl_2]$. The benzohydrazide (L) in these complexes depicts monodentate behavior, providing terminal amino nitrogen as a donor atom. Compared to inactive precursors (free benzohydrazides and Pd^{2+}), almost all Pd(II) complexes showed *in vitro* antiglycation activity, illustrating the potential role of resulting complexes in the suppression of diabetes and related disorders. The presence of free carbonyl group in complexes has been recognized as possible cause of antiglycation. This study also indicated Pd(II) compounds as far more superior inhibitors of urease and phosphodiesterase-I than parent ligands; many of them exhibited inhibitions equivalent or even greater than the standard inhibitors (thiourea, urease; EDTA, phosphodiesterase), which shows their potential use in future in the control of peptic ulcer and arthritis, respectively. The structure activity relationship (SAR) study demonstrated that complexation, steric hindrance, position of substituents, electron density around metal centre, hydrogen bonding and coordination mode of complexed ligands play prime role in modulating the biological activities of complexes.

Keywords: Pd(II) complexes, Benzohydrazides, Structure elucidation, Spectroscopy, Enzyme inhibition, Protein glycation

Introduction

The development of Pd(II)-based drugs has received much importance after the success of cisplatin in the cancer therapy, due to considerable resemblance in the coordination chemistry of Pt(II) and Pd(II) [1–3]. Pd(II) complexes with variety of ligands have demonstrated an immense potential to exploit in the medication as shown by their diversified bioactivities: antitumor [4], antimalarial [5], antituberculosis [6], antiprotozoal [7], anti-HIV [8], anticonvulsant [9] and antioxidant [10]. However, there is a great scarcity of literature concerning other biological activities such as enzyme inhibition and antidiabetic properties of Pd(II) complexes, particularly with nitrogen donor ligands; it provided us enough momentum to ensue in this direction. For current study, urease, phosphodiesterase-I and glycation were selected to inhibit by Pd(II) complexes of hydrazides.

Ureases are widely occurring, cysteine rich, nickel dependant enzymes, which catalyze urea hydrolysis producing ammonia and carbon dioxide as final products [11]. The excessive urease activity in soil results in decreased efficiency of soil fertilization with urea (owing to ammonia volatilization) and root damage (owing to increase in soil pH) [12]. Urease is also a key enzyme utilized by bacterium *Helicobacter pylori*, which constitutes a virulence factor in human infections, such as chronic gastritis, peptic ulceration, duodenitis, gastric cancer and urinary stone formation [13–15]. Therefore, the discovery of safe and potent urease inhibitors is an important area of pharmaceutical, agronomic and environmental research. Urease inhibitors are regarded as a suitable target for therapeutic interventions to eradicate *H. pylori* infections, acting as new antiulcer drugs [16], and they may also be helpful in the field of urea based commercial fertilizers [17].

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Nucleotide pyrophosphatases/Phospho-diesterases (NPPs, ecto-NPPs or PDs-I) are composed of a group of multi-gene family of metalloenzymes, which catalyze the release of nucleoside-5′-monophosphates from hydrolysis of a variety of nucleotide derivatives [18]. NPPs are found in a wide variety of organisms and various tissues, for example, distributed in mammalian intestinal mucosa, liver, blood, brain capillaries, slivary gland epithelium, testis and uterus [18–20]. Phosphodiesterases are known to be involved in osteoarthritis, tumor cell motility and insulin resistance in type II diabetes [21]. NPPI plays a crucial role in normal and pathological mineralization or calcification of bone and cartilage; therefore, the inhibition of NPPIs can be useful in treatment for some forms of arthritis [22].

Glycation is a non-enzymatic spontaneous reaction between reducing sugar and coexisting protein. This reaction undergoes formation of labile Schiff base that rearranges to a stable Amadori product, which is followed by a complex cascade of reactions leading to the formation of advanced glycation end products (AGEs) [23–26]. Glycation depends on the generation of reactive oxygen species through trace amounts of redox active metal ions [27] and on the degree and duration of hyperglycemia *in vivo* [28]. The accumulation of tissue AGEs together with enhanced oxidative stress has shown an important role in the progression of aging and diabetic complications, including retinopathy, neuropathy, atherosclarosis, nephropathy, embryopathy and delayed healing of wounds [29–33]. The failure of existing antidiabetic drugs necessitates the discovery of new inhibitors of protein glycation to have a long term solution for the management of diabetes and age-related diseases.

This report is focused on synthesizing and evaluating the antiurease, antiphosphodiesterase-I and antiglycating behaviors of Pd(II)-benzohydrazide complexes to seek their effects in the control of peptic ulcer, arthritis and diabetes related disorders, respectively. To the best of our knowledge, this paper presents first systematic antiglycation and enzyme (urease, phosphodiesterase) inhibition study reported so far on any nitrogen donating hydrazide-Pd(II) complex with positive outcomes.

Experimental

General Experimental Procedures

The reagent grade chemicals, purchased from Merck, BDH or Sigma Aldrich, were utilized without further purification for current study. All the solvents were distilled before use. Distilled water was further passed through deionizer (ELGA Cartridge Type C114). A Perkin Elmer 2400 series II CHN/ S analyzer was employed in order to carry out CHN (elemental) analysis. EI-mass spectra of ligands were obtained from a Finnigan MAT 311-A apparatus. FT-IR spectra at 400-4000 cm^{-1} were recorded on KBr disks employing a Shimadzu 460 IR spectrophotometer. A Bruker 300 spectrometer was utilized to record ^{1}H-NMR and ^{13}C-NMR spectra in DMSO at 300 or 400 MHz using TMS as internal standard. Effective magnetic moments of powdered complexes were determined at room temperature using sealed-off calibrant solution of $MnCl_2$ by using a Sherwood MSB Mk1 magnetic susceptibility balance. A Hanna (HI-8633) conductivity meter was used to measure conductance. Pd content of complexes was calculated *via* EDTA titration using sodium nitrite as a selective masking agent in the presence of xylenol orange indicator [34]. The IC_{50} values for inhibition of urease, phosphodiesterase and protein glycation were calculated using EZ-fit Enzyme Kinetics Program (Parella Scientific, Amherst, USA).

Synthesis of Hydrazide Ligands

Fourteen benzohydrazide ligands (**1**–**14**) were synthesized according to Scheme-1 using our previously reported method [10]. The spectral and elemental data for characterization of ligands **6**–**14** have already been reported by us [10, 35], while the characterization data of remaining ligands (**1**–**5)** are intended to present here (given below). EI-mass fragmentation patterns of ligands **1**–**5** are also shown in Scheme-2.

2-Chlorobenzohydrazide **(1)**. ^{1}H-NMR (400 MHz, DMSO): δ 7.49-7.36 (*m*, 4H, H-3/H-4/H-5/H-6); ^{13}C-NMR (400 MHz, DMSO): δ 165.58, 135.62, 130.81, 130.28, 129.55, 129.10, 126.97; EI MS m/z (rel. Abundance, %): 170 (29, M^{+}), 155 (5), 139 (100), 121 (4), 111 (60), 87 (13), 75(47), 63 (16), 50 (60); Anal. Calcd. for $C_7H_7N_2OCl$: C 49.26, H 4.10, N 16.41; found: C 49.61, H 3.95, N 16.55.

3-Chlorobenzohydrazide **(2)**. ^{1}H-NMR (400 MHz, DMSO): δ 7.83 (*s*, 1H, H-2), 7.77 (*d*, 1H, J = 7.2 Hz, H-6), 7.57 (*d*, 1H, J = 6.0 Hz, H-4), 7.48 (*t*, 1H, J = 7.6 Hz, H-5); ^{13}C-NMR (400 MHz, DMSO): δ 164.28, 135.26, 133.13, 130.86, 130.29, 126.74, 125.59; EI MS m/z (rel. Abundance, %): 170 (15, M^{+}), 139 (100), 111 (75), 87 (2), 75 (50), 61 (4), 50 (57); Anal. Calcd. for $C_7H_7N_2OCl$: C 49.26, H 4.10, N 16.41; found: C 49.35, H 3.96, N 16.46.

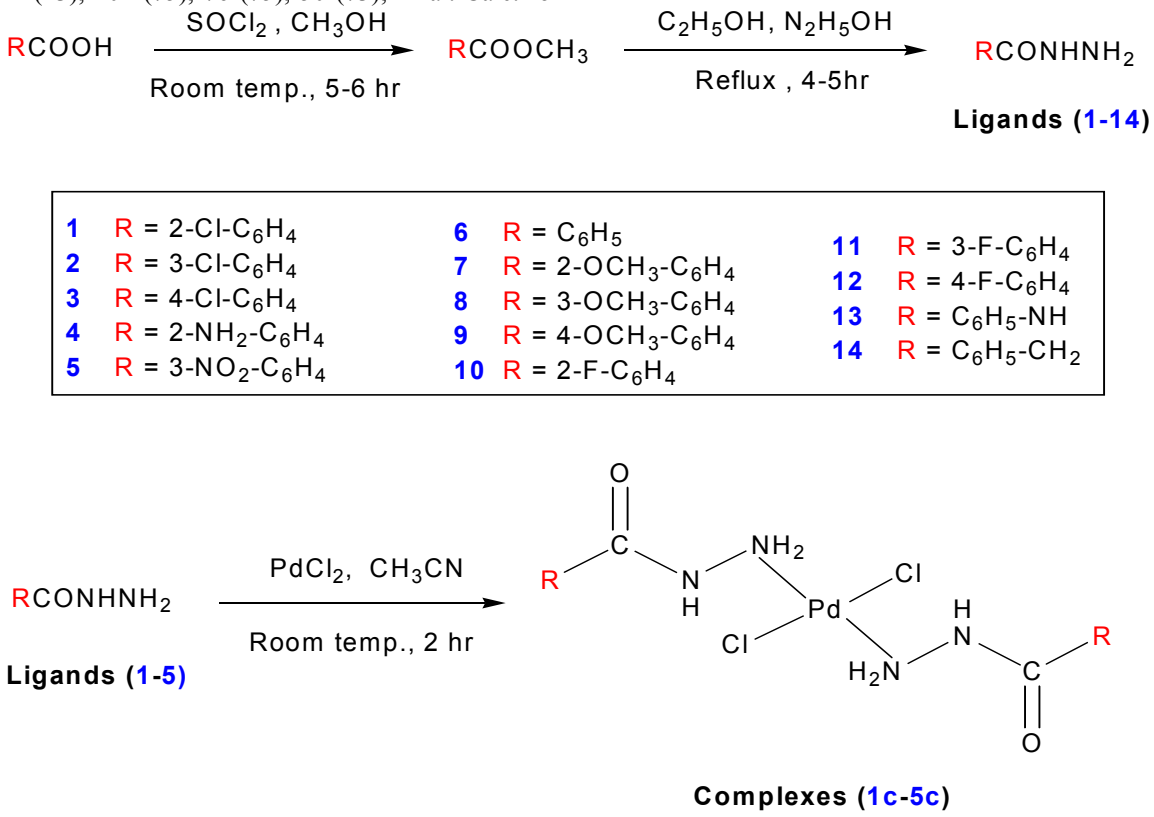
4-Chlorobenzohydrazide **(3)**. ^{1}H-NMR (300 MHz, DMSO): δ 7.82 (*d*, 2H, *J* = 8.7 Hz, H-2/H-6), 7.51 (*d*, 2H, *J* = 8.4 Hz, H-3/H-5); ^{13}C-NMR (300 MHz, DMSO): δ 164.74, 135.82, 132.02, 128.82, 128.37; EI MS m/z (rel. Abundance, %): 170 (30, M^{+}), 139 (100), 111 (78), 87 (2), 75(47), 61 (3), 50 (27); Anal. Calcd. for $C_7H_7N_2OCl$: C 49.26, H 4.10, N 16.41; found: C 49.64, H 3.93, N 16.61.

2-Aminobenzohydrazide **(4)**. ^{1}H-NMR (400 MHz, DMSO): δ 7.40 (*d*, 1H, *J* = 6.8 Hz, H-6), 7.10 (*t*, 1H, *J* = 6.8 Hz, H-4), 6.67 (*d*, 1H, *J* = 8.0 Hz, H-3), 6.46 (*t*, 1H, *J* = 7.2 Hz, H-5); ^{13}C-NMR (400 MHz, DMSO): δ 168.46, 149.29, 131.46, 127.57, 116.15, 114.60, 113.68; EI MS m/z (rel. Abundance, %): 151 (25, M^{+}),121 (9), 120 (100), 92 (54), 65 (54), 52 (9); Anal. Calc. for $C_7H_9N_3O$: C 55.62, H 5.96, N 27.81; found: C 55.80, H 6.15, N 27.92.

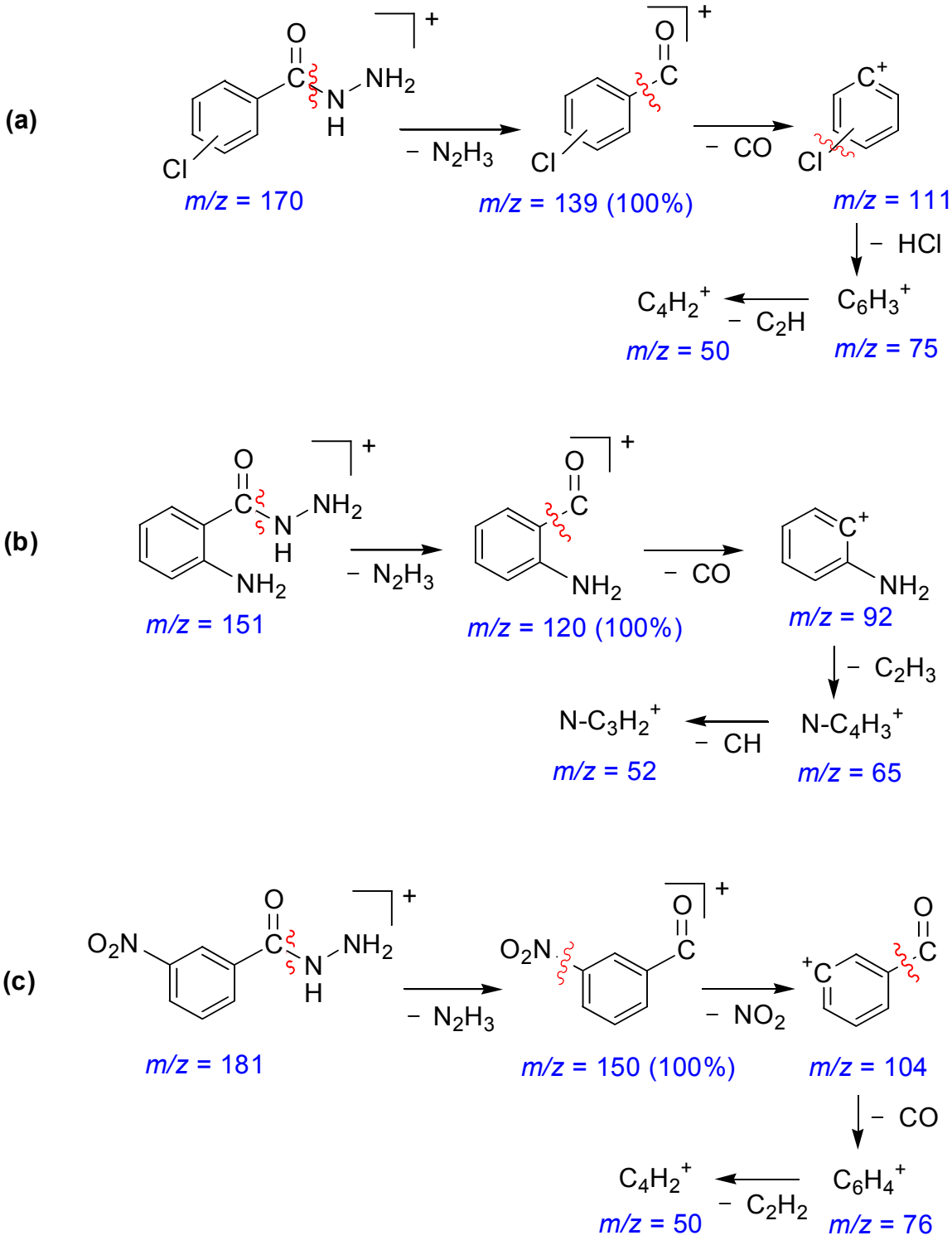
3-Nitrobenzohydrazide **(5)**. ^{1}H-NMR (400 MHz, DMSO): δ 7.67 (*s*, 1H, H-2), 8.35 (*dd*, 1H, *J* = 8.5 Hz, *J* = 1.6 Hz, H-4), 8.24 (*dd*, 1H, *J* = 7.0 Hz, *J* = 1.2 Hz, H-6), 7.58 (*t*, 1H, *J* = 8.0 Hz, H-5); ^{13}C-NMR (400 MHz, DMSO): δ 163.49, 147.75, 134.71, 133.18, 130.09, 125.62, 121.72; EI MS m/z (rel. Abundance, %): 181 (50, M^{+}), 151 (35), 150 (100), 122 (13), 104 (75), 76 (75), 50 (73); Anal. Calc. for $C_7H_7N_3O_3$: C 46.40, H 3.87, N 23.20; found: C 46.84, H 3.67 N, 23.48.

Synthesis of Palladium(II)-Hydrazide Complexes

The equal volume (10 ml) solutions of Pd(II) chloride (1 mmol) and hydrazide **1**–**5** (2 mmol) in acetonitrile were mixed slowly in the presence of a drop of concentrated HCl. The resulting mixture containing yellow precipitates was kept on stirring at room temperature for about 2 hr in order to complete the reaction. The yellow precipitates were then filtered, washed with acetonitrile and finally dried in vacuum to afford complexes **1c**–**5c**. Various physical and chemical measurements, including conductivity, magnetic susceptibility, CHN analysis, Pd content determination and spectral (^{1}H-NMR, ^{13}C-NMR, FT-IR) analysis were utilized for structural elucidation of Pd(II) complexes. The characterization data of complexes **1c**–**5c** are presented in Table-1 to 3. The synthesis and characterization of Pd(II) complexes **6c**–**14c** (obtained from hydrazides **6**–**14**, Scheme-1) have already been published by us [10, 35].



Scheme-1: Structure and synthetic route for Pd(II) complexes of hydrazides



Scheme-2: EI-mass fragmentation patterns of ligands **1**–**3** (**a**), **4** (**b**) and **5** (**c**) with mass to charge ratios (*m/z*). The fragment showing 100% peak intensity (*m/z* = 139, **a**; *m/z* = 120, **b**; *m/z* = 150, **c**) is most stable. The other identified fragments of **1**–**3** (**a**) were different in relative peak heights; *m/z* values were similar. The red wavy lines show the possible point of fragmentation from where the bond breaks

Urease Inhibition Assay

The previously reported indophenol assay [36–37] was adopted with slight modifications for urease inhibition study. The reaction mixture comprising 20 µl of jack bean urease solution (1U/well) and 50 µl of buffer containing 200 mM urea was incubated with 10 µl of DMSO solution of test compound at 30°C for 15 min in 96-well plates. Afterward, 60 µl phenolic reagent (0.005% w/v sodium nitroprusside and 1% w/v phenol) and 60 µl of alkali reagent (0.1% active chloride, i.e., NaOCl, and 0.5% w/v NaOH) were added to each well. The absorbance at 625 nm was measured against the control after 1 hr, using a microplate reader (Spectra Max, Molecular Devices, USA) spectrophotometer. All reactions were carried out in triplicates in a final volume of 200 µl. The assay was performed at pH 6.8 (10 mM sodium phosphate). Thiourea was used as a standard urease inhibitor. The percentage inhibitions were calculated from the following formula:

Percent enzyme inhibition = [{Absorbance $_{(control)}$ − Absorbance $_{(sample)}$} / Absorbance $_{(control)}$]*100

Phosphodiesterase Inhibition Assay

The inhibition activity against snake venom phosphodiesterase-I was determined using previously reported method [38–39] with slight modifications. To measure inhibition activity, a reaction mixture comprising 85 µl of 33 mM Tris-HCl buffer (pH 8.8), 40 µl of 30 mM Mg-acetate, 40 µl of sample in DMSO (in varying concentrations), 30 µl of enzyme (0.000742 U/well) and 120 µl of 0.33 mM of bis-(*p*-nitrophenyl) phosphate was incubated at 37˚C in 96-well microplates. After 30 min, the absorbance was measured at 410 nm, using a Spectra Max 340 reader. Ethylenediammine tetraacetic acid, EDTA, was employed as a positive control. The data for each sample were collected in triplicates. The percent inhibition values were calculated by similar formula as used for urease inhibition assay.

Antiglycation Assay

For *in vitro* antiglycation assay, the method described by Rahber *et al.,* 2003 [40] and Lo *et al.,* 1993 [41] was utilized with slight modifications. This assay involves testing out of inhibition of methyl glyoxal (MGO) mediated glycation of bovine serum albumen (BSA) using fluorometry. Briefly, triplicate sets of solutions each comprising 50 µl BSA (10 mg/ml in phosphate buffer), 14 mM MGO (50 µl), 0.1 M phosphate buffer (pH 7.4, containing 30 mM NaN_3) and 20 µl test sample (prepared in DMSO) were incubated under aseptic conditions in 96-well plates at 37°C for 9 days. After incubation, every sample was analyzed for the development of specific fluorescence intensity (excitation at 330 nm and emission at 440 nm) against sample blank. This measurement was carried out on a microtitre plate reader (Spectra Max, Molecular Devices, USA) spectrophotometer. Rutin was employed as positive control. To calculate the percent inhibition of formation of AGEs for each inhibitor, the following formula was used:

Percent inhibition = [1– (fluorescence $_{(sample)}$ / fluorescence $_{(control)}$)]*100

Results and Discussion

Physico-Chemical Properties

All Pd(II)-hydrazide complexes were yellow, amorphous and non hygroscopic. These complexes were fairly stable at room temperature with high solubility in DMF, DMSO and THF; however, they were poorly soluble in other organic solvents such as ethanol, acetone and ethyl acetate.

Low effective magnetic moments (μ_{eff}) of 0.113–0.312 B.M. (Table-1) and sharp peaks in the NMR spectra of Pd(II) complexes indicated their diamagnetic nature and hence square planar geometry [42]. Low molar conductivity values (2.75–9.89 $\Omega^{-1}cm^2mol^{-1}$) of freshly prepared solutions of Pd(II) complexes in DMSO suggested their non electrolytic nature [43]. The addition of $AgNO_3$ solution in fresh DMSO solutions of complexes produced white precipitates of AgCl, showing the presence of chloride in the complexes. CHN and Pd contents of all Pd(II) complexes were consistent with general formula $[PdL_2Cl_2]$. Hence, Pd(II) ion in each complex is coordinated with two monodentate benzohydrazide (L) ligands and two chloride ions, forming a square planar complex (Scheme-1).

IR Spectroscopy

The vibrational data of hydrazides (**1**–**5**) and their Pd(II) complexes (**1c**–**5c**) are presented in Table-2. The FT-IR spectra of ligand **1** and its complex **1c** have shown in Fig. 1. The terminal hydrazinic amino group in hydrazides exhibits a pair of fairly intense peaks at 3185–3443 cm^{-1}, which is attributed to amino N-H symmetric and asymmetric stretching modes [44]. A considerable shift of 22–102 cm^{-1} in these bands observed upon coordination with metal center in **1c**–**5c** suggests the coordination of hydrazinic terminal amino nitrogen with Pd(II)

[45]. A sharp peak ranging from 3014 to 3037 cm^{-1} for ligands **1**–**5** is assigned to hydrazinic imino group [44], which was shifted to higher frequencies in complexes **1c**–**5c** due to coordination of neighboring amino group with palladium(II). The carbonyl group of hydrazides exhibits a stretching frequency at 1620–1672 cm^{-1} [46], which was slightly shifted upto ± 15 cm^{-1} in Pd(II) complexes, indicating noncoordination of carbonyl oxygen with Pd(II). The bands for NH_2 bending and C-N, C-O, N-N and aromatic C=C stretching modes are also assigned and given in Table-2 [47–52]. The FT-IR study supports the monodentate behavior of all the benzohydrazides, which provide terminal hydrazinic amino nitrogen as possible coordinating site.

NMR Spectroscopy

Table-3 provides the ^{1}H-NMR and ^{13}C-NMR spectral data for the ligands (**1**–**5**) and corresponding Pd(II) complexes (**1c**–**5c**) in fresh DMSO solutions. Fig. 2 and 3 compare the ^{1}H-NMR and ^{13}C-NMR spectra, respectively, for ligand **5** and its complex **5c**.

Table-1: Physical and analytical data of Pd(II)-hydrazide complexes (**1c**–**5c**)

Comp.	Molar mass	% Yield	μ_{eff} [B.M.]	Molar conductance $[\Omega^{-1}cm^2\ mol^{-1}]$	Elemental analysis Cal (Found) %			
					Pd	C	H	N
1c	518.61	88.32	0.113	7.31	20.52 (20.71)	32.39 (32.58)	2.70 (2.79)	10.80 (10.99)
2c	518.61	85.15	0.312	5.48	20.52 (20.85)	32.39 (32.68)	2.70 (2.44)	10.80 (11.00)
3c	518.61	75.31	0.182	3.11	20.52 (20.67)	32.39 (32.73)	2.70 (2.39)	10.80 (11.07)
4c	479.42	85.81	0.125	2.75	22.20 (22.53)	35.04 (35.40)	3.75 (3.42)	17.52 (17.81)
5c	539.72	78.18	0.173	9.89	19.72 (20.05)	31.14 (31.39)	2.59 (2.43)	15.57 (15.98)

Table-2: Comparison of FT-IR data of hydrazides (**1**–**5**) and their Pd(II) complexes (**1c**–**5c**)

Comp.	NH_2	Imino NH	C=O	NH_2 Bending	C=C	C-N	C-O	N-N	Other vs
1	3286, 3186	3023	1646	1593	1518	1337	1128	952	894, 754, 720, 655, 463, 436
1c	3251, 3164	3050	1658	1591	1551	1328	1162	910	781, 745, 676, 618, 523, 462
2	3304, 3219	3028	1664	1619	1559	1341	1119	997	892, 804, 724, 690, 551, 464
2c	3276, 3185	3102	1655	1606	1567	1332	1161	919	758, 733, 671, 520, 466
3	3310, 3214	3014	1661	1617	1558	1346	1095	988	882, 840, 729, 624, 532, 450
3c	3280, 3192	3022	1650	1594	1537	1323	1093	1011	845, 748, 658, 590, 528, 495
4	3443, 3324	3026	1620	1578	1507	1319	1261	957	795, 746, 662, 506, 448
4c	3445, 3350	3058	1614	1582	1542	1312	1250	908	755, 669, 616, 510, 434, 409
5	3276, 3208	3073	1672	1630	1533	1346	1142	993	851, 814, 717, 670, 593, 481
5c	3202, 3119	3075	1657	1616	1529	1352	1225	931	812, 713, 665, 501, 432

All values are in cm^{-1}

Table-3: Comparison of NMR data of hydrazides (**1**–**5**) and their Pd(II) complexes (**1c**–**5c**)

Compound	^{1}H-NMR [δ ppm]			^{13}C-NMR [δ ppm]	
	NH_2	NH	Aromatic protons	C=O	Aromatic carbons
1	4.47	9.53	7.36 - 7.49	165.58	126.97 - 135.62
1c	5.60	9.78	7.39 - 7.49	165.60	126.95 - 131.02
2	4.51	9.87	7.46 - 7.83	164.28	125.59 - 135.26
2c	5.85	10.14	7.50 - 7.85	164.35	125.72 - 133.30
3	4.49	9.84	7.50 - 7.84	164.74	128.37 - 135.82
3c	5.85	10.09	7.52 - 7.84	165.10	128.46 - 133.72
4	4.34	9.42	6.45 - 7.40	168.46	113.68 - 149.29
4c	6.52	7.71	7.05 - 7.45	168.46	114.72 - 150.10
5	4.62	10.15	7.74 - 8.63	163.49	121.72 - 147.75
5c	7.05	10.83	7.76 - 8.64	163.61	121.89 - 147.77

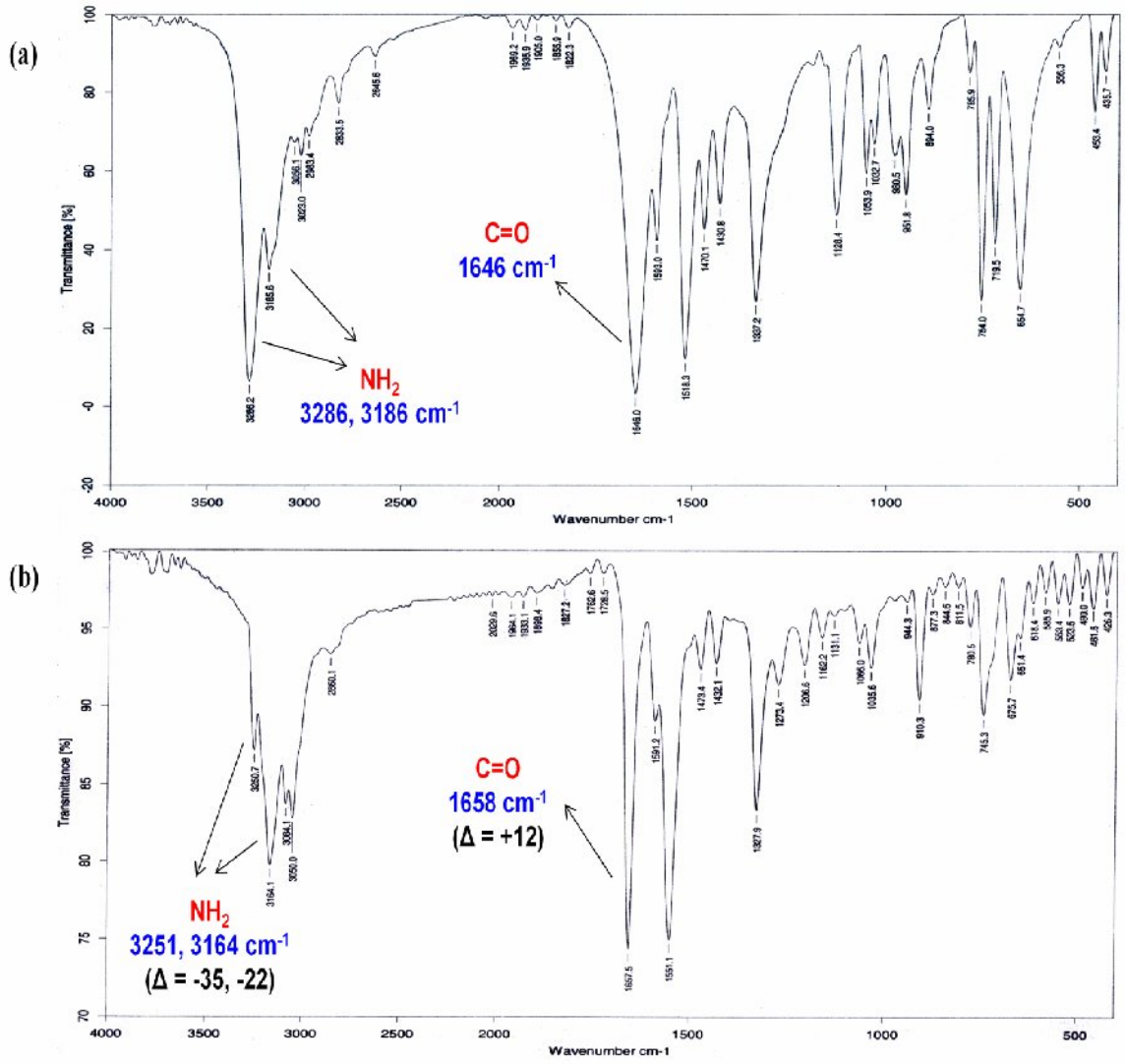


Fig. 1: FT-IR spectrum of ligand **1** (**a**) and its Pd(II) complex **1c** (**b**). A significant decrease (35 and 22 cm^{-1}) in the two amino stretching frequencies compared to small shift (12 cm^{-1}) in the carbonyl stretching frequency after complexation suggests coordination of amino group and noncoordination of carbonyl group with Pd(II).

The signals at 4.34–4.62 ppm exhibited by terminal $–NH_2$ group of ligands in the ^{1}H-NMR spectra were shifted downfield by 1.13–2.43 ppm in complexes, suggesting their coordination with Pd(II) [45]. A singlet at 9.42–10.15 ppm for **1-5** is attributed to imino NH group. The imino protons in the ligands were 0.47–1.75 ppm less shifted than amino protons after complexation; this demonstrates the absence of direct coordination of imino nitrogen with Pd(II) [53]. The appearance of imino NH signal for both ligands and complexes suggests the presence of ketone (protonated) form and hence neutral state of both free and coordinated hydrazides in DMSO, which further supports the absence of direct linkage of imino nitrogen with Pd(II) in complexes [44, 53]. The resonances of aromatic protons appeared at 6.45–8.63 ppm for ligands **1**–**5** [51, 54], which were slightly shifted downfield at 7.05–8.64 ppm in complexes, owing to increased conjugation upon complexation [55]. The signal due to amino substituent attached to phenyl ring of hydrazide in compounds **4** and **4c** appeared near aromatic region at 6.30 and 6.67 ppm, respectively [45]. The ^{1}H-NMR spectral considerations are consistent with IR results in supporting the coordination of $–NH_2$ group with Pd(II).

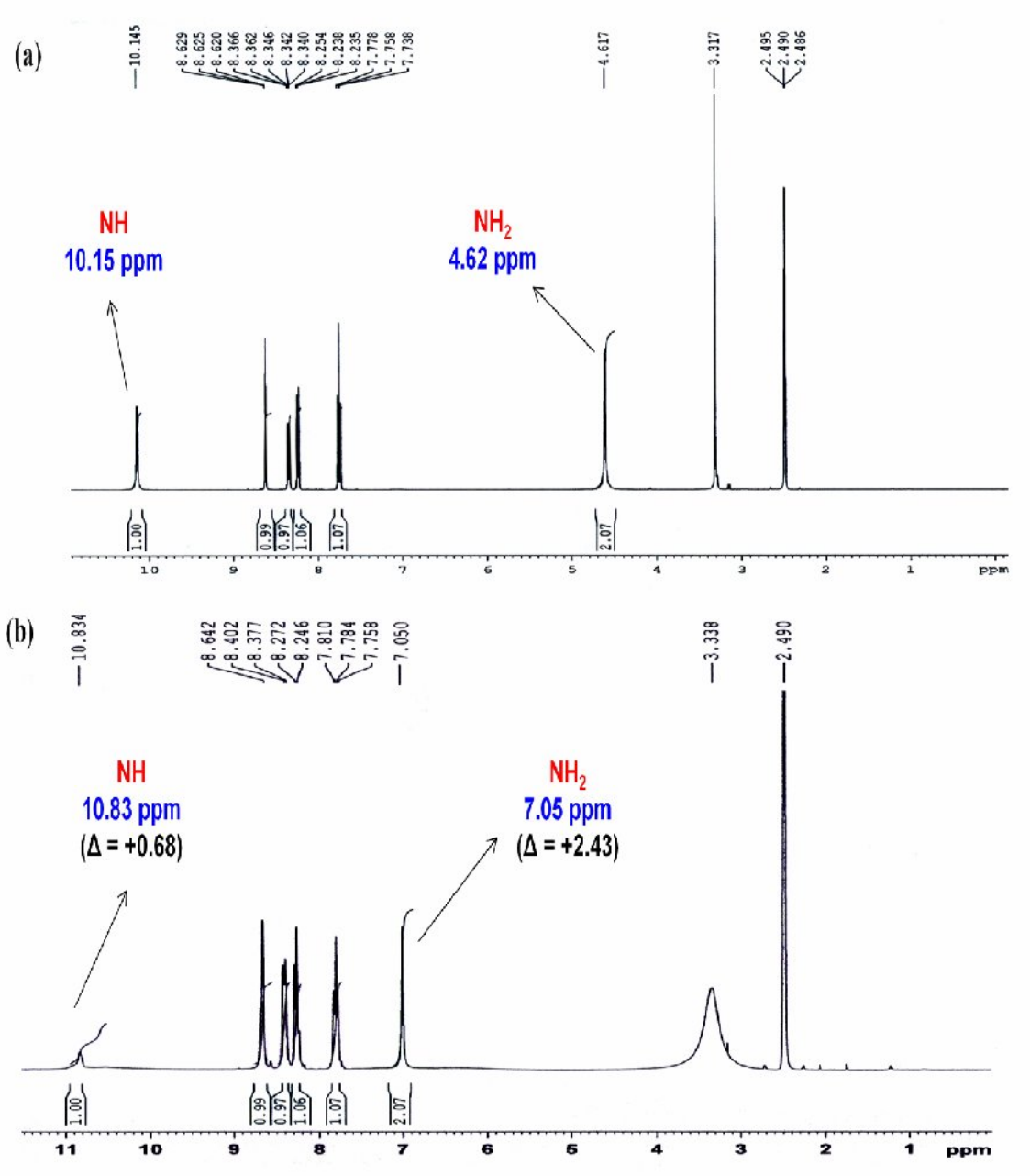


Fig. 2: ^{1}H-NMR spectrum of ligand **5** (a) and its Pd(II) complex **5c** (b). A considerable shift of 2.4 ppm in the signal of amino protons compared to very small shift for other protons indicates the coordination of amino group with Pd(II)

The ^{13}C-NMR spectral results agreed well with the bonding modes predicted from IR and ^{1}H-NMR spectral studies. The number of carbon peaks observed for ligands and respective complexes were in accord with expected values. The aromatic carbon atoms in ligands show signals at 113.68–149.29 ppm [56], which exhibited a slight shift upon complexation and appeared at 114.72–150.10 ppm. The most downfield signal (at 163.49–168.46 ppm) in ligands **1**–**5** is exhibited by carbonyl carbon [57]; it remained almost same (Δ = ± 0.36 ppm) for corresponding complexes **1c**–**5c**, which excludes the possibility of direct linkage of carbonyl oxygen with Pd(II) in complexes.

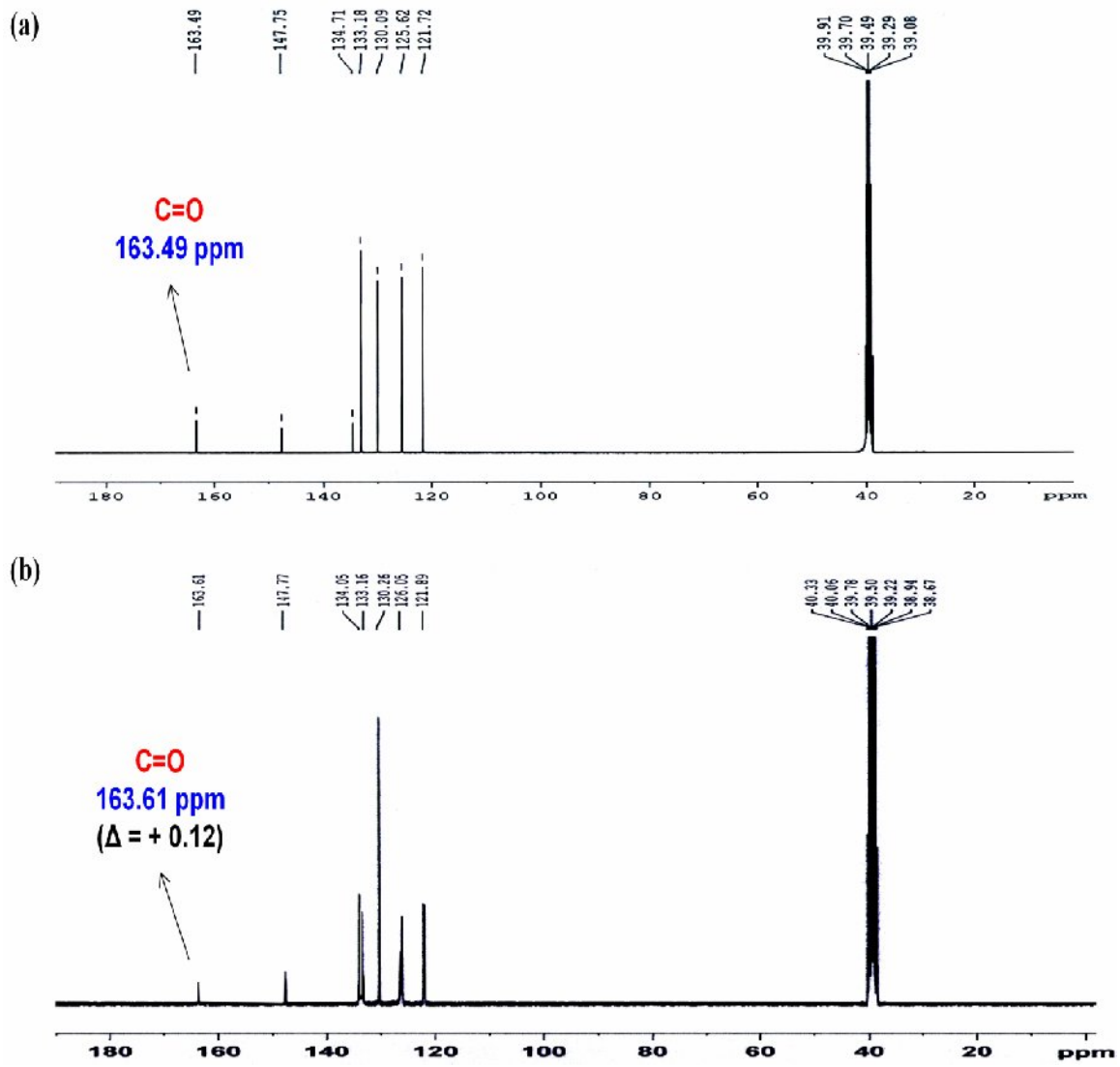


Fig. 3: ^{13}C-NMR spectrum of ligand **5** (a) and its Pd(II) complex **5c** (b). A negligible shift of 0.12 ppm in the carbonyl carbon signal suggests the absence of coordination of carbonyl group with Pd(II)

Pd(II)-hydrazide complexes did not show any cis-trans isomerization in fresh DMSO solutions, manifested by their NMR spectra (Fig. 2 and 3). In the NMR spectra of Pd(II)-hydrazide complexes, a single set of peaks was observed for particular functionalities (corresponding to a single isomer) instead of a pair of peaks (corresponding to a mixture of cis and trans isomers). This indicates that the Pd(II)-hydrazide complexes are sufficiently stable in solution form and prevent any cis-trans isomerization in fresh DMSO solution. To maintain minimum steric hindrance between two hydrazides in the complex and hence greater stability, the configuration of hydrazides in complexes is proposed to be trans [58]. The trans geometry can also be justified by poor solubility of benzohydrazide complexes in polar solvents such as water, methanol and acetonitrile.

Urease Inhibition Activity

Fourteen Pd(II) hydrazide complexes (**1c**–**14c**) along with corresponding uncoordinated ligands (**1**–**14**) were examined *in vitro* against Jack-bean urease by Berthelot alkaline phenol-hypochlorite method [36]. This assay is based on the decrease in the color of indophenol blue pigment (λ_{max} = 625nm) produced upon reaction of NH_4^+ with hypochlorite ion in the presence of phenol and sodium nitroprusside catalyst at slightly alkaline conditions [37]. The results are presented in terms of IC_{50} values in Table-4.

Table-4: Enzyme (Urease, phosphodiesterase-I) inhibition and antiglycation activities of hydrazides [a] and their Pd(II) complexes **1c**– **14c**

Compound	Urease	Phosphodiesterase-I		Antiglycation
	IC_{50} (µM) ± SEM	IC_{50} (µM) ± SEM	% Inhibition ± SEM	IC_{50} (µM) ± SEM
1c	254 ± 1.25	Precipitated	6.80 ± 0.008	451 ± 1
2c	331 ± 2.54	Precipitated	- 6.24 ± 0.003	385 ± 7
3c	Precipitated	Precipitated	- 0.93 ± 0.001	398 ± 2
4c	127 ± 0.98	446 ± 1.56	17.20 ± 0.011	NA
5c	>750	Precipitated	- 3.70 ± 0.002	428 ± 7
6c	61 ± 0.55	Precipitated	10.00 ± 0.005	473 ± 7
7c	278 ± 1.72	Precipitated	- 10.74 ± 0.002	426 ± 4
8c	458 ± 2.14	Precipitated	1.59 ± 0.001	407 ± 1
9c	224 ± 1.84	Precipitated	- 6.57 ± 0.001	334 ± 10
10c	456 ± 2.32	175 ± 1.07	25.45 ± 0.052	469 ± 1
11c	595 ± 3.12	307 ± 1.13	43.69 ± 0.092	394 ± 4
12c	325 ± 1.88	148 ± 0.95	44.65 ± 0.008	425 ± 4
13c	140 ± 1.15	146 ± 1.05	44.86 ± 0.072	590 ± 13
14c	98 ± 1.32	350 ± 1.85	25.02 ± 0.009	714 ± 10
$PdCl_2$	4 ± 0.05	Precipitated	10.75 ± 0.004	NA
Thiourea [b]	57 ± 1.01			
EDTA [c]		295 ± 1.00	30 ± 0.075	
Rutin [d]				295 ± 2

[a] All hydrazides were inactive against urease, phosphodiesterase-I and protein glycation
[b] Standard inhibitor of urease
[c] Standard inhibitor of phosphodiesterase
[d] Standard inhibitor of protein glycation
SEM = Standard error of mean
NA = Not active.

All free hydrazides were negative for urease inhibition. In contrast, Pd(II)-hydrazide complexes (except **5c** and **3c**) were active in showing urease inhibition with IC_{50} values ranging from 61 µM to 595 µM compared to thiourea (Standard inhibitor, IC_{50} = 57 µM). Compound **5c** (having 3-nitro group) was only poorly active (IC_{50} > 750 µM), while for complex **3c** (containing 4-chloro substituent) it was unable to obtain IC_{50} due to solubility issues and precipitation of the complex under reaction conditions at higher concentrations. The Pd(II) complexes showed dose-dependent inhibition of urease; the inhibition activity profiles for the compounds are provided in Supplementary Material 1. A considerable amount of work has also been reported in literature concerning urease inhibition by other metal complexes, particularly those of organotin(IV), V(IV), Cu(II), Zn(II), Co(II), Ni(II), Mn(III) and Cu(II)-Zn(II) complexes with different ligands [59–62].

The precursor metal salt ($PdCl_2$) was a most potent urease inhibitor (IC_{50} = 4 µM) having about 15 times more inhibitory potential compared to thiourea. This indicates the significant role of Pd^{2+} centre in urease inhibition. Several other heavy metal ions, including Ag^{+}, Hg^{2+}, Bi^{3+}, Cu^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, Pb^{2+} and Mn^{2+}, in addition to alkali metal ions, have also been reported a long time before to show strong urease inhibition efficacies [63–69]. This property of heavy metal ions can be used to produce inexpensive and sensitive urease-based sensors to detect heavy metals [70].

Although the antiurease activity of free Pd(II) ion was significantly higher than the respective complexes, the Pd(II)-hydrazide complexes may be regarded as the better inhibitors than free Pd(II) from pharmacological stand point to develop antiulcer drugs. This consideration is based on the potential ability of ligands to reduce the toxicity of free metal ion [71]. For example, Kojima *et al.,* 2002 reported that the chelating ligands can reduce the toxicity of free Zn(II) ion [72]. However, cytotoxic studies are essential to confirm the relative toxicity of free Pd(II) ion and respective hydrazide complexes.

Ureases are the sulfhydryl enzymes containing multiple cysteine residues and two nickel ions per active site in the enzyme [73]. The inhibition of urease by Pd(II) compounds could be expected either due to replacement of enzymatic Ni^{2+} by Pd^{2+} (softer Lewis acid belonging to same group in the Periodic Table) or due to coordination of Pd^{2+} with softer Lewis bases (cysteinyl sulfur or histidine nitrogen) in the enzyme. The later mechanism has also been suggested previously to occur for Zn(II), Cu(II) and Bi(III) ions [62, 65]. The hydrazides in Pd(II) complexes may involve hydrogen bonding with enzymatic side chain residues through carbonyl or NH groups, which may restrict the active site flap, leading to enzyme inactivation [74]. However, detailed investigations are needed to confirm the actual mechanism of urease inhibition.

Among Pd(II) complexes, the one having simplest unsubstituted benzohydrazide (i.e., **6c**) had the highest antiurease activity (IC_{50} = 61 µM), which was comparable to thiourea. The incorporation of

different substituents on phenyl ring in Pd(II)-hydrazide complexes, as in case of halo (**1c**, **2c**, **10c**–**12c**), amino (**4c**), nitro (**5c**) and methoxy (**7c**–**9c**) substituted complexes, reduced the urease inhibition power by at least 66 μM to greater than 690 μM compared to parent complex **6c**. This may be attributed to poor interaction of substituted groups with surrounding amino acid residues in the enzyme active site. The Pd(II) complexes having NH group (**13c**) or CH_2 group (**14c**) between carbonyl and benzene ring of hydrazide moiety exhibited good inhibitory potential bearing IC_{50} values of 140 μM and 98 μM, respectively. The absence of substituent on phenyl ring and the presence of CH_2 or NH groups attached to ring in complexes could contribute in decreasing steric hindrance near the metal centre. The absence of steric factors may facilitate the entrance of inhibitor in the pocket of enzyme active site and may avoid its unfavorable interactions with surrounding amino acids, providing better inhibition of enzyme.

The complexes with *meta* substituents on phenyl ring (**2c**, 3-chloro; **8c**, 3-methoxy; **11c**, 3-fluoro) were weaker urease inhibitors (ΔIC_{50} = 77–270 μM) as compared to corresponding *ortho* (**1c**, 2-chloro; **7c**, 2-methoxy; **10c**, 2-fluoro) and *para* (**9c**, 4-methoxy; **12c**, 4-fluoro) substituted complexes. This may be attributed to the specific configuration at the enzyme active site, which may interact less favorably with *meta* substituted complexes.

There were number of observations which strongly emphasized that the high electron density on the complex strengthens the antiurease potential. For example, the Pd(II) complex with highly electron donating amino substituent (**4c**, IC_{50} = 127 μM) had much higher antiurease activity (ΔIC_{50} > 623 μM) than strongly electron withdrawing nitro substituted complex (**5c**). Similarly, the *ortho*- or *para*-substituted chloro (**1c**), methoxy (**7c** and **9c**) and fluoro (**10c** and **12c**) complexes (which may increase electron density on metal centre inductively) were stronger urease inhibitors (ΔIC_{50} = 77–270 μM) compared to respective *meta*-compounds: **2c** (chloro), **8c (**methoxy**)** and **11c** (fluoro). Furthermore, the replacement of CH_2 group of compound **14c** with electron withdrawing NH group (as in compound **13c**) resulted in decrease in the antiurease potential (increase in IC_{50} by 42 μM). Hence, the high electron density strongly favors the urease inhibition, and this property will be used to design future urease inhibitors with an optimized activity profile.

The incorporation of hydrophobic CH_2 group in compound **14c** caused an increase of 37 μM in the IC_{50} and hence decreased urease inhibition efficacy compared to parent complex **6c**. The report of Amtul *et al.,* 2002 also describes that the introduction of hydrophobic groups or increase in carbon chain length may reduce the urease inhibition power of a compound [75].

In short, the urease inhibition may involve coordination of Pd(II) with cysteinyl sulfur or histidine nitrogen atoms of urease, and the extent of inhibition may be controlled by various factors including the presence of metal centre, electron density, steric hindrance, hydrophobicity, and nature and position of substituents. This study provides useful information to synthesize optimized Pd(II)-based urease inhibitors that would be further used to develop effective antiulcer drugs and to solve the problems associated with urea fertilization.

Phosphodiesterase Inhibition Activity

Fourteen hydrazide ligands (**1**–**14**) and respective Pd(II) complexes (**1c**–**14c**) were investigated for their inhibition against snake venom phosphodiesterase-I (NPPI). The assay was based on monitoring the production of yellow colored *p*-nitrophenol (λ_{max} = 400 nm) from bis-(*p*-nitrophenyl) phosphate by NPPI action [39]. All the uncoordinated ligands were found inactive. Only six complexes (**4c**, **10c**–**14c**) showed promising NPPI inhibition, and their IC_{50} values are presented in Table-4. These six complexes illustrated the inhibition of NPPI in a dose-dependent manner; NPP1 inhibition activity profiles for complexes are available as Supplementary Material 2. The remaining eight complexes (**1c**–**3c**, **5c**–**9c**) and free Pd(II) did not enable the calculation of IC_{50} due to their insolubility or turbidity at higher concentrations under assay conditions. Therefore, to compare the inhibitory effect and to evaluate structure activity relationships, the results of NPPI inhibition activity are presented in terms of percent inhibition values at 120 μM for all compounds.

In contrast to inactive free hydrazides, the Pd(II) compounds showed varying degree of NPPI inhibition. For example, three complexes (**10c**, **12c** and **13c**) were excellent NPPI inhibitors revealing IC_{50} values significantly lower (ΔIC_{50} = 120 μM, **10c**; 147 μM, **12c**; 149 μM, **13c**) compared to EDTA (standard inhibitor, IC_{50} = 295 μM). The inhibition activity of compound **11c** was almost equivalent (having a small difference of 12 μM in IC_{50}) to that of standard, while the complexes **4c** and **14c** demonstrated moderate inhibition (IC_{50} values were higher with a difference of 55–151 μM) compared to EDTA. The rest of the complexes showed either

week inhibitory action (≤ 10% inhibition) or inactivity (negative % inhibition) compared to EDTA (30.11% inhibition).

As the parent ligands were inactive, this study identifies the important role of Pd(II) centre in phosphodiesterase inhibition by Pd(II) complexes. Previous studies also describe the key role of other metal ions such as Cd(II), Pb(II), Cu(II) and V(IV) in the inhibition of various types of phosphodiesterases [76–78]. The NPPs are known to contain extracellular cysteine rich somatomedin-B-like domains, which mediate the dimerization of NPPs through disulfide bonds [79]. The inhibition of NPP1 by Pd(II) compounds is expected due to coordination of sulfur atoms of cysteine residues with Pd(II). The Hg(II) ion in *p*-chloromercuribenzoate has been documented earlier to inhibit venom phosphodiesterase by similar mechanism [80]. The inhibition of NPP1 by Pd(II) compounds may be important in the suppression of pathological calcification of bones or chondrocalcirosis, the condition developed by over expression of NPP1 [22].

The precursor metal salt ($PdCl_2$) was a moderate inhibitor (10.75% inhibition) of NPP1 compared to EDTA (30.11% inhibition). The complexation of Pd(II) with inactive hydrazides may enhance or decline the inhibition potency of Pd(II) depending upon the structure of hydrazides coordinated. For example, Pd(II) complexes having 3-fluoro (**11c**) or 4-fluoro (**12c**) substituent and that having NH group in between carbonyl group and benzene ring (**13c**) exhibited much better inhibition powers (43.69%, 44.65% and 44.86 %, respectively) than precursor Pd(II) and even higher than EDTA. Similarly, the complex with CH_2 group between carbonyl group and benzene ring (**14c**), or the complexes which possess 2-fluoro group (**10c**) or 2-amino substituent (**4c**) on phenyl ring, also revealed higher inhibitory potentials (Δ% inhibition was 6.45–14.70%) compared to precursor metal ion. However, these three complexes (**4c**, **10c** and **14c**) showed lower inhibition activity than EDTA with a difference of 4.55–12.80% in percent inhibition. The inhibition potency of Pd(II) remained almost same when it was complexed with unsubstituted benzohydrazide (**6**). In contrast, the coordination of Pd(II) with remaining ligands, which possess chloro (**1c**–**3c**), methoxy (**7c**–**9c**) or nitro (**5c**) substituents, made the complexes either totally inactive or slightly active inhibitors of NPPI, as indicated by their negative or low percent inhibition values (1.59–6.80%), respectively. Hence, fine tuning of ligand structure may be used to synthesize Pd(II) complexes with optimized NPPI inhibitory potentials to develop antiarthritis drugs.

The results clearly indicated that the fluoro substitution on phenyl ring in benzohydrazide-Pd(II) complexes (e.g., **10c**, **12c**) plays an important role in NPPI inhibition activity. This could be attributed to small size and high electronegativity of fluorine, which may contribute in decreasing steric hindrance and creating hydrophilic or H-bonding interactions with enzyme. These results are in agreement with the previous study of Zheng *et al.,* 2008, wherein the introduction of fluoro groups enhanced inhibitory potential of phenyl alkyl ketones against phosphodiesterase 4 (PDE4) [81]. The significant NPP1 inhibition activity of **13c** (having 149 µM lower IC_{50} than standard) and **14c** (having 55 µM higher IC_{50} than standard) may also be due to decreased steric hindrance near the metal centre, offered by NH (**13c**) or CH_2 (**14c**) moiety present in between carbonyl group and benzene ring. It strengthens the hypothesis that steric hindrance in a compound would play a dominant role in the suppression of NPPI inhibition activity.

The strongly electron donating amino substituent (as in **4c**) enhanced the percent inhibition of parent complex **6c** from 10.00 to 17.20%. In contrast, the strongly electron withdrawing nitro group (as in **5c**) reduced the percent inhibition of **6c** to - 3.7%. Thus, high electron density on a compound may also play important role in enhancing NPP1 inhibition.

Excluding amino and fluoro complexes (**4c**, **10c**–**11c**), all substituted Pd(II) complexes (chloro, **1c**–**3c**; nitro, **5c**; methoxy, **7c**–**9c**) showed weaker inhibitory effect (negative to less than 7% inhibition) compared to reference complex **6c**. Therefore, substitution may also affect NPPI inhibition to some extent. Shortly, the phosphodiesterase I (NPPI) inhibition may be modulated by complexation with Pd(II), position of substituents, steric hindrance, hydrogen bonding and electronic effects.

Antiglycation Activity

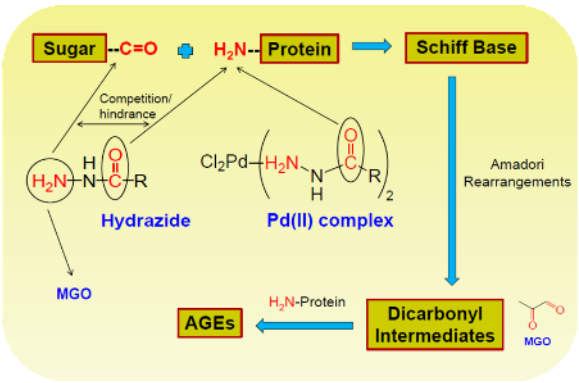
The antiglycation activity of fourteen hydrazides (**1**–**14**) and their Pd(II) complexes (**1c**–**14c**) was evaluated using high throughput biochemical (BSA-MGO fluorescence) assay [40]. MGO is a reactive dicarbonyl intermediate of protein glycation, which can react with albumin protein to form fluorescent protein-carbonyl adducts, providing *in vitro* method to examine protein glycation [41].

The results of antiglycation assay are presented in Table-4.

The precursors (free hydrazides and $PdCl_2$) showed inactivity towards inhibition of glycation. In fact, free Pd(II) had negative percent inhibition value, indicating the involvement of free Pd(II) in the activation of glycation rather than its inhibition. However, the combination of individually inactive hydrazide and Pd(II) ion in a single molecule (metal complex) resulted in acquiring antiglycation potential in almost all cases. Only complex **4c** was inactive. Other complexes demonstrated a varying degree of antiglycation activity from potent to weak, bearing IC_{50} values from 334 to 714 µM compared to rutin (standard inhibitor, IC_{50} = 295 µM). It is therefore suggested that complexation may play a significant role in reducing the glycation related toxicity of free Pd^{2+} ion and in increasing the antiglycation potential of the precursor entities.

The amino and carbonyl groups in hydrazides are suggested as highly critical in the inhibition of glycation. The amino group may block glycation by reaction with reactive carbonyl intermediates similar to guanidine, a standard glycation inhibitor [82]. The carbonyl group may also prevent glycation by trapping amino groups of protein. Since a hydrazide molecule [R-CO-NH-NH_2] has both amino and carbonyl groups, it should show antiglycation potential, but surprisingly all free hydrazide ligands were inactive. This can be explained as follows: there may be a competition between carbonyl group and amino group (or relative affinity) in a same hydrazide molecule towards amino group of protein and carbonyl group of methylglyoxal, respectively. Furthermore, a relatively small hydrazide molecule, having small distance between carbonyl and amino groups, may remain unable to accommodate simultaneously both methylglyoxal and a large protein molecule to form a bis-Schiff base. These two factors (i.e., competition between carbonyl and amino group, and small size of hydrazide) may possibly prevent a hydrazide to block active site on either protein or MGO. Thus, a free hydrazide ligand plays no role in antiglycation.

Since the carbonyl group of coordinated hydrazide is free while the amino group having coordinated with Pd(II) ion in all Pd(II)-hydrazide complexes (Scheme-1), the positive antiglycation activity of Pd(II) complexes (except for **4c**) is expected due to the engagement of active amino group of protein with free carbonyl group of complexes. The complex **4c** possesses an additional amino group (as a phenyl substituent) along with free carbonyl group; therefore, the inactivity of complex **4c** can be explained by similar reason as described for uncoordinated hydrazides. It may also be due to the steric hindrance observed by *ortho* substitution of amino group. The possible mechanistic interactions of hydrazides and their Pd(II) complexes during antiglycation process have been shown in Scheme-3.



Scheme-3: Probable antiglycation mechanistic interactions of hydrazides and their Pd(II) complexes. The hydrazides do not inhibit the formation of advanced glycation end products (AGEs) due to simultaneous competitive (restricted) interaction of their NH_2 and C=O groups with MGO (methyl glyoxal, a glycation intermediate used for *in vitro* antiglycation assay) and protein. The Pd(II)-hydrazide complexes (having only free carbonyl group) are antiglycating probably due to their facile interaction with protein's amino group

When phenyl ring of reference complex **6c** (no substituent, IC_{50} = 473 µM) was substituted with chloro (**1c**–**3c**), nitro (**5c**), methoxy (**7c**–**9c**) or fluoro (**10c**–**12c**) groups, the antiglycation power was significantly improved with decrease in IC_{50} of 22–88 µM for **1c**–**3c**, 45 µM for **5c**, 47–139 µM for **7c**–**9c** and 4–79 µM for **10c**–**12c** compared to **6c**. The halo or oxygen containing groups may facilitate the interaction of Pd(II) complexes with protein by increasing hydrophilicity and hydrogen bonding. Secondly, these groups may also increase the electrophilicity of neighboring carbonyl group in Pd(II)-hydrazide complexes *via* negative inductive effect, facilitating the nucleophilic attack by amino group of protein. It may result in a Schiff base adduct formation between protein and Pd(II) complex more favorably and hence more inhibition of MGO-mediated protein glycation. It is therefore deduced that the substituents containing electronegative atoms may enhance antiglycation effectiveness. In contrast,

the incorporation of NH or CH_2 moiety (in between carbonyl group and benzene ring) in **6c** caused a significant decrease in the antiglycation efficacy; the percent increase in IC_{50} was 24.73% for NH moiety (**13c**) and 50.95% for CH_2 moiety (**14c**). It is suggested that the NH moiety in complexes may involve in intramolecular H-bonding with neighboring carbonyl group, while CH_2 moiety may decrease the electrophilicity of carbonyl carbon for amino group of protein, both resulting in less favorable interaction of carbonyl group with protein and hence reduced antiglycation ability of **13c** and **14c** compared to **6c**.

The results also demonstrated a strong SAR with position of substituents on coordinated hydrazides. All *ortho*-substituted Pd(II) complexes (**1c**, 2-chloro; **7c**, 2-methoxy; **10c**, 2-fluoro) were comparatively weaker inhibitors of glycation (percent increase in IC_{50} was 4.2–21.6%) than respective *meta*- and *para*-substituted complexes: **2c** (*meta*-chloro, ΔIC_{50} = 66 μM), **3c** (*para*-chloro, ΔIC_{50} = 53 μM), **8c** (*meta*-methoxy, ΔIC_{50} = 18 μM), **9c** (para-methoxy, ΔIC_{50} = 92 μM), **11c** (*meta*-fluoro, ΔIC_{50} = 75 μM) and **12c** (*para*-fluoro, ΔIC_{50} = 44 μM). This emphasizes the important role of steric hindrance in antiglycation activity. In short, slight modification in the structure of Pd(II)-hydrazide complexes may optimize antiglycation efficacies due to change in electronic and steric properties. The effective inhibition of glycation process would be an efficient tool to delay the ongoing diabetic complications.

Conclusions

The current study presents the synthesis of square planar Pd(II)-benzohydrazide complexes of general formula $[PdL_2Cl_2]$, wherein amino hydrazinic nitrogen coordinates with Pd(II) ion in trans configuration. The subsequent *in vitro* biological screening demonstrates that complexation of benzohydrazides with Pd(II) makes the complexes superior as compared to free ligands against urease, phosphodiesterase-I and protein glycation. This study first time identifies some Pd(II) complexes of nitrogen donor hydrazides as promising inhibitors of urease (e.g., **6c**, **14c**), phosphodiesterase-I (e.g., **10c**–**13c**) and protein glycation (e.g., **2c**, **9c**) with inhibition efficacies comparable to that of standards (thiourea, EDTA and rutin, respectively). The development of effective Pd(II)-based antiglycating agents and inhibitors of urease and phosphodiesterase make them interesting drug candidates for the treatment of diabetes, peptic ulcer and arthritis, respectively; therefore the identified Pd(II) complexes deserve to be researched further in this field. Furthermore, the enzyme inhibition activity of Pd(II) complexes is positively related to hydrophilicity and presence of NH/CH_2 moiety, while negatively related to steric hindrance near the metal centre. Other factors such as the presence of fluoro groups, binding mode of coordinated ligand and position of phenyl substituents in Pd(II) complexes may also strongly influence their profile of enzyme inhibition and antiglycation. These properties will be used in future to design optimized metal based inhibitors of urease, phosphodiesterase-I and glycation.

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Conflict of Interest

There is no potential conflict of interest declared by any author.

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